



## **Growth promotion in pigs by oxytetracycline coincides with down regulation of serum inflammatory parameters and of hibernation-associated protein HP-27**

**Soler, Laura; Miller, Ingrid; Hummel, Karin; Razzazi-Fazeli, Ebrahim; Jessen, Flemming; Escibano, Damian; Niewold, Theo**

*Published in:*  
Electrophoresis

*Link to article, DOI:*  
[10.1002/elps.201500529](https://doi.org/10.1002/elps.201500529)

*Publication date:*  
2016

*Document Version*  
Peer reviewed version

[Link back to DTU Orbit](#)

### *Citation (APA):*

Soler, L., Miller, I., Hummel, K., Razzazi-Fazeli, E., Jessen, F., Escibano, D., & Niewold, T. (2016). Growth promotion in pigs by oxytetracycline coincides with down regulation of serum inflammatory parameters and of hibernation-associated protein HP-27. *Electrophoresis*, 37(10), 1277-1286.  
<https://doi.org/10.1002/elps.201500529>

---

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

**Growth promotion in pigs by oxytetracycline coincides with down regulation of serum inflammatory parameters and of hibernation-associated protein HP-27**

Laura Soler<sup>1, #</sup>, Ingrid Miller<sup>2</sup>, Karin Hummel<sup>3</sup>, Ebrahim Razzazi-Fazeli<sup>3</sup>, Flemming Jessen<sup>4</sup>, Damian Escibano<sup>5</sup>, Theo Niewold<sup>1,\*</sup>

<sup>1</sup> Livestock-Nutrition-Quality Division, Department of Biosystems, Faculty of Biosciences Engineering, KU Leuven, Kasteelpark Arenberg 30, Heverlee 3001, Belgium.

<sup>2</sup> Department of Biomedical Sciences, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria.

<sup>3</sup> VetCore Facility for Research, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria.

<sup>4</sup> Division of Industrial Food Research, National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

<sup>5</sup> Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence “Campus Mare Nostrum”, University of Murcia, 30100 Espinardo, Murcia, Spain

\*Corresponding author: T.A. Niewold, phone +3216 321560, fax +3216321994, email [theo.niewold@biw.kuleuven.be](mailto:theo.niewold@biw.kuleuven.be)

<sup>#</sup> L. Soler is currently working at INRA Val de Loire, Nouzilly, France.

**List of abbreviations:**  $\alpha$ 1AG, Alpha 1-acid glycoprotein; AGP, antimicrobial growth promoters; AHSG,  $\alpha$ -2-HS-glycoprotein; ApoF, apolipoprotein F, CLU, clusterin; Hp, haptoglobin; FRAP, ferric reducing anti-oxidant power; HP, hibernation associated protein; HP-27, hibernation associated plasma protein; OTC, oxytetracycline; PON1, paraoxonase type-1; SAA, serum amyloid A

**Keywords:** Proteomics; Immunometabolism; Oxytetracycline; Antimicrobial growth promoters; hibernation-associated plasma protein-27.

Total number of words including figure and table legends: 5916

## **Abstract**

The growth promoting effect of supplementing animal feed with antibiotics like tetracycline has traditionally been attributed to their antibiotic character. However, more evidence has been accumulated on their direct anti-inflammatory effect during the last two decades. Here we used a pig model to explore the systemic molecular effect of feed supplementation with sub therapeutic levels of oxytetracycline (OTC) by analysis of serum proteome changes. Results showed that OTC promoted growth, coinciding with a significant down regulation of different serum proteins related to inflammation, oxidation and lipid metabolism, confirming the anti-inflammatory mechanism of OTC. Interestingly, apart from the classic acute phase reactants also down regulation was seen of a hibernation associated plasma protein (HP-27), which is to our knowledge the first description in pigs. Although the exact function in non-hibernators is unclear, down regulation of HP-27 could be consistent with increased appetite, which is possibly linked to the anti-inflammatory action of OTC. Given that pigs are good models for human medicine due to their genetic and physiologic resemblance, the present results might also be used for rational intervention in human diseases in which inflammation plays an important role such as obesity, type 2 diabetes and cardiovascular diseases.

## 1 Introduction

Immunometabolism is a relatively new field of study that investigates the interplay between the metabolic state and the immune system. Relatively recently, it was realized that obesity promotes inflammation, predisposing for a host of conditions such as cardiovascular disease [1, 2]. Thought to be important in this process is the up regulation of the metabolic or postprandial inflammation the intensity of which is related to the energy value, glycemic index and lipid profile of food [3, 4]. Similar to humans with unhealthy habits, intensively produced animals such as chicken and pigs ingest large amounts of (high) energy feed, causing a state of inflammation too [5, 6]. The latter leads to a suboptimal feed utilization, muscle catabolism, decreased appetite, and consequently diminished growth [4]. Interestingly, the growth retardation in livestock used to be remedied by the use of the nowadays (in the EU) banned antimicrobial growth promoters (AGP) as feed additives. This suggested that they act as anti-inflammatory agents [5], although the conventional theory was that they worked by an antibiotic mechanism [7]. The latter is quite unlikely, mainly because of the low, sub therapeutic concentrations used, and a direct anti-inflammatory mechanism for growth promotion by AGP was proposed [5]. Among the most effective AGP were macrolides, shown to have also in humans direct anti-inflammatory effects on the immune system in lung disease [8]. Cyclines were recently shown to have clear anti-inflammatory effects on macrophages *in vitro* [9, 6], and in mice [10] and broilers [6], which in the latter was paralleled by growth promotion. There are also studies which point at a similar mechanism in pigs for cyclines [11].

Pigs are better experimental models for human research than chicken (or mice), due to their genetic, anatomic and physiologic resemblance, in particular concerning the gastrointestinal tract, and therefore more relevant for metabolic studies related to human

immunometabolism [12]. Interestingly, antibiotic use in children has recently been associated with increased body weight gain [13]. In the present study, we aimed at investigating the mechanism behind growth promotion induced by a cycline in pigs. The serum proteome of pigs supplemented in feed with oxytetracycline (OTC) was compared with that of unsupplemented pigs by 2DE DIGE followed by MALDI-TOF/TOF. In addition to complete serum, fractions of the same sera enriched for less abundant proteins by three different methods were analyzed in the same way. After validation, interpretation of results was performed through a bioinformatics systems biology analysis of the regulated proteins. Results were [interpreted together](#) with growth rates and clinical biochemistry parameters to define the systemic effect of OTC supplementation in pigs.

## **2 Materials and methods**

### *Animals*

The experimental animal protocol was approved by the KU Leuven Ethics Committee for Animal Experiments (protocol approval number P047/2008). Four week-old weaned piglets (Piètrain x Hypor) were housed in one stable, containing six 2 m<sup>2</sup> pens, slatted floors with bedding, separated by bars, containing five piglets each. Piglets were weighed, and divided over the pens as equally as possible taking litter, weight, and sex into account. Temperature was maintained at 26-27° C, and piglets were kept at a 16 h light, 8 h dark cycle. Commercial starter and piglet meal diets (NV D2000 baby starter and duo starter, Roeselare, Belgium) were offered to the animals from 28 to 47 d and from 48 to 65 d of age, respectively, containing vitamins and minerals meeting or exceeding National Research Council requirements. Feed and water were provided *ad*

*libitum* throughout the experiment. The experimental period lasted 37 days in which control piglets (n=15) received only the commercial diet, whereas treated animals (n=15) received a diet containing a commercial in-feed preparation of OTC (courtesy of Huvepharma, Antwerp, Belgium) mixed in their feed with a final concentration of 200 ppm OTC. Piglets were weighed at days 0, 32 and 65 of the test period. During the whole experiment the health status of the animals was monitored by visual and physical examination. Two animals from control group and one animal from OTC group were excluded from the study as they showed disease symptoms such as lameness, weight loss and diarrhea. At test day 37, blood was collected by jugular venipuncture, and allowed to clot for 1 hour at room temperature and was then centrifuged at 2000 x g for 15 min to obtain serum.

#### *Acute phase protein determination*

Serum levels of haptoglobin (Hp) and serum amyloid A (SAA) were used to determine the general inflammatory status of the studied animals (n=27). Haptoglobin was measured with a colorimetric commercial kit (Tridelta Development Ltd, Kildare, Ireland) according to the manufacturer's instructions, and SAA was determined with a competitive ELISA assay previously developed and validated [14].

#### *Sample material for proteomic analysis*

Four serum samples of each group were randomly selected from control and OTC-supplemented piglets. All of those specimens were subjected to one-dimensional SDS-PAGE or 2DE DIGE, both in non-fractionated form and after a depletion/enrichment step. The latter methods were used for reduction of major serum protein levels to

facilitate uncovering differences in low abundant serum proteins. The following approaches were used:

*Method 1: Albumin and IgG Depletion:* A commercial kit (ProteaPrep Albumin and IgG Depletion Sample Prep Kit; Protea, Morgantown, WV, USA) was used which employs an non-antibody based ligand-ligand interaction for capture of serum albumin and IgG, thus suitable for its use in non-human species. A volume of 10 µL of serum was subjected to this depletion with the ProteaPrep Kit following the manufacturer's instructions. Besides the fall-through fraction containing the enriched material, also bound proteins were collected by elution with 400 µL 0.1M glycine-HCl pH 2.5 (into tubes containing Tris buffer for pH adjustment), for further testing. Fall-through and bound proteins were kept at -20°C until analysis.

*Method 2: Protein enrichment on hexapeptide resin:* the ProteoMiner protein enrichment system (Bio-Rad, Hercules, CA, USA) is based on the use of a combinatorial peptide binding library, which affinity-captures and amplifies the low-abundance proteome [15]. A total amount of 10 mg (approx. 250 µL of crude serum) of protein was treated according to the manufacturer's instructions. Both eluted and bound fractions were kept for analysis. The depleted serum (the initially bound fraction of this approach) was dialyzed against 100 mM ammonium acetate pH 7 in a PlusOne mini 1kDa cut-off dialysis kit (GE Healthcare, Munich, Germany), lyophilized and kept at -20°C. Samples were later dissolved in IPG sample buffer [16] before further analysis.

*Method 3: Triton X-114 cloud point separation:* This in-house protein depletion method enriches more hydrophobic proteins. It consists of a phase separation with Triton X-114



where the detergent rich phase is collected after raising the temperature to above the cloud point. The enriched fraction is then diluted at a temperature below the cloud point and concentrated by a TCA-acetone precipitation step. Pellets were dissolved in appropriate sample buffer and kept at -20°C until analysis. Starting material for this method was 400 µL of serum [17].

The protein concentration of fractions obtained by the above methods was determined according to Bradford [18], and composition further analyzed as described below.

### *Proteome analysis*

#### *One-dimensional electrophoresis*

A first screening and comparison of protein patterns of all different samples was achieved by one-dimensional SDS-PAGE under reducing conditions on 140 x 140 x 1.5 mm gradient gels (10–15% T, 2.7% C) in a Hoefer SE 600 vertical electrophoresis chamber (Hoefer scientific instruments, San Francisco, CA, USA). For serum samples, 0.5 µl serum were applied per lane and protein bands stained with colloidal Coomassie Blue G-250 (Novex, Invitrogen, Carlsbad, CA, USA) [19]. For the less concentrated fractions from depletion/enrichment experiments, 2.5 µg protein per lane were separated in SDS-PAGE and subjected to silver staining [20]. Gels were scanned in a GE Imagescanner III (GE Healthcare, Munich, Germany). All the images were digitalized and analyzed by Image Studio Lite Software (Li-Cor Biosciences, Lincoln, NE, USA).

#### *2DE DIGE*

Differences in the serum proteome (or their enriched fractions) of control and OTC piglets were evaluated by 2-DE DIGE. Samples were minimally labelled with CyDye DIGE™ fluorescent dyes according to the manufacturer's instructions (GE Healthcare,

Munich, Germany). To exclude any effect of possible preferential labelling, dye swap was performed. An IPG-DALT system was employed for 2DE, as described previously [16]. This includes first-dimensional electrophoresis in IPG strips of 10 cm length followed by SDS-PAGE as described above. Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare, Munich, Germany). Image analysis including spot detection, matching, normalization and quantification was performed in four data sets. Spots of interest were identified after statistical analysis (see below) and were excised from the gel after MS-compatible silver staining [16].

#### *MALDI-TOF/TOF analyses*

Sample preparation for mass spectrometry was performed as recently described [21]. Briefly, spots of interest were washed, destained, reduced and alkylated before in-gel tryptic digestion (TrypsinGold, Mass Spectrometry Grade, Promega, Madison, WI, USA). Extracted dried peptides were desalted and spotted prior to data acquisition on a MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics, Bremen, Germany) in MS and MS/MS mode. Processed spectra were searched via an in-house Mascot server (Matrix Science, Boston, MA) in the SwissProt/UniProt (Release 2014\_06) or EST\_mammals (EST\_120) database using the following search parameters: taxonomy: *sus scrofa*; enzyme: trypsin/semi-trypsin, global modifications carbamidomethylation on cysteine; variable modifications: Deamidated (NQ), Gln->pyro-Glu (N-term Q), Oxidation (M); MS tolerance 100 ppm; MS/MS tolerance 1 Da; one missed cleavage allowed. Identifications were considered statistically significant where  $P < 0.05$ .

#### *Interpretation of results through systems biology analysis*

Regulated proteins were listed as official gene names and subjected to systems biology analysis using the “Set Distiller” module of GeneDecks website [22]. Because far more human genes are annotated and more information in databases is available for humans than for pigs, the human database was used for this functional analysis. Regulated proteins were assigned to a specific pathway and retrieved when significant ( $P < 0.05$ ).

#### *Confirmatory methods*

[Protein regulation results](#) from 2DE DIGE analysis were [validated](#) by running [the following](#) additional tests, all with unfractionated serum:

*Serum total IgG determination:* Total IgG was quantified by using a pig-specific ELISA test (USCN Life Sciences, Hubei, China), following the manufacturer’s instructions.

*Alpha 1-acid glycoprotein ( $\alpha 1AG$ ) measurement:* The relative abundance of  $\alpha 1AG$  was determined by immunoblotting by using an in-house anti-bovine  $\alpha 1AG$  rabbit polyclonal antibody [23] with cross-reactivity with pig. Diluted serum samples (1/10) as well as pure bovine  $\alpha 1AGP$  (1 $\mu$ g) as positive control were separated on 4-12% SDS-PAGE minigels and blotted onto PVDF membrane.  $\alpha 1AG$  was detected by a biotinylated anti- $\alpha 1AG$  polyclonal antibody and an HRP-Streptavidin conjugate (Sigma, Saint-Louis, MO, USA) with the help of a chromogen (4-chloro-1-naphthol) [20].

Subsequent total protein staining of the membrane with Coomassie brilliant blue R-250 served as a loading control. Both images were evaluated by scanning on a GE Imagescanner III (GE Healthcare, Munich, Germany). All the images were digitalized and analyzed by Image Studio Lite Software (Li-Cor Biosciences, Lincoln, NE, USA).

For each lane,  $\alpha$ 1AG band intensity values were normalized relative to the total protein staining.

*Biochemical measurement of paraoxonase type-1 (PON1) activity:* PON1 was quantified by determining the serum arylesterase activity using p-nitrophenyl acetate as substrate in an automated clinical chemistry analyzer (Olympus AU2700, Olympus Diagnostica, Tokyo, Japan) using a method adapted for pig serum samples [24]. Intra-assay and inter-assay CV ranges were 0.5% to 1.5% and 4.8% to 5.5%, respectively. Serial dilution of 2 serum samples resulted in linear regression equations with correlation coefficients close to 1 ( $r = 0.998$  and  $0.994$ ). The assay detection limit was 0.15 U/L.

*Total serum antioxidant activity:* The total antioxidant capacity of the serum samples studied was determined through the ferric reducing anti-oxidant power (FRAP) assay as described elsewhere [25].

#### *Statistical analysis*

Statistical analysis of body weight measurements, acute phase protein determination and validation results were performed by using the software program GraphPad Prism 5 for Windows (GraphPad software, La Jolla, CA, USA). Differences in values of each parameter described above were analyzed by two-tailed Student's t-tests, as Kolmogorov-Smirnov test results indicated Gaussian distribution for all datasets. The significance level was set at  $P < 0.05$  in all cases. DeCyder software Version 7.0 (GE Healthcare, Munich, Germany) was used to identify differentially regulated spots ( $P < 0.05$ ; Fold-change  $\pm 1.3$ ) by two-way ANOVA.

### 3 Results

#### *Effect of OTC supplementation on weight gain and serum levels of acute phase proteins*

Supplementation of animal feed with OTC at sub-therapeutic concentrations was associated with significantly higher weight gain of supplemented animals at the end of the experimental period compared to controls (Table 1A). OTC supplementation also resulted in numerically lower levels of serum haptoglobin and significantly lower levels of serum amyloid A (Table 1A).

#### *Enrichment of minor proteins*

Protein content and 1DE profile of initial serum samples were similar (CV% of protein concentration was 13.05%; Fig.1A). Banding patterns of enriched samples were distinct from serum, but typical for the respective method, and with little difference between individual samples of each group (for Proteominer and ProteaPrep treatment, see Fig.1B and 1C). Not enough material was obtained after Triton X-114 cloud point separation to additionally screen individual samples in SDS-PAGE (Fig.1D). Recoveries of enriched samples ranged between 0.1% of initial protein content for the Triton X-114 cloud point separation to 6.9% for Proteominer and 38% for ProteaPrep depletion systems. No differences in recovery were found between treatment groups.

#### *2DE DIGE*

Combining results from all 2DE analyses, differences in spot intensity between gels from control and OTC-supplemented animals were identified in eighty-one matched different spots ( $P < 0.05$ ). The Proteominer method yielded only 5 differentially regulated spots, all of which were identified as immunoglobulin heavy chains based on

earlier observations. These were not subjected to MS analysis, and left out in the further analysis. For some of the regulated spots the amount of protein was insufficient to confidently identify the present proteins by MALDI-TOF/TOF. In total, thirteen different proteins were identified out of thirty-one spots (Table 2, Fig. 2, and Supplemental Table 1). Among those were two faint spots identified as  $\alpha$ -2-HS-glycoprotein (AHSG) which were clearly smaller size breakdown products: in MS analysis for spot #2259 only peptides of the amino-terminal part of the protein were found, for spot #1376 only from the C-terminus (Fig. 2C). Analysis of the spots of the intact AHSG chain (spots #328-331; Fig 2A) showed no differential regulation. Clusterin (CLU) appears as two distinct spot chains in pig serum, but was in the present study too faint for positive MS identification. In the Triton X-114 cloud point separation method 2 CLU containing spots were identified (Fig. 2D), spot #715 showed only peptides from the  $\alpha$ -chain, spot #758 only from the  $\beta$ -chain. Both spot chains were regulated in a similar way, but with different degree of significance. From all spots a single protein was identified except spot #484 in which apart from PON1 also peptides from  $\alpha$ 1AG were found. Together, the two depletion methods identified three additional proteins not detected in whole serum (hibernation associated plasma protein (HP-27), PON1, and apolipoprotein F (ApoF)) as well as additional fragments of AHSG,  $\alpha$ 1AG, and Hp, with similar trends but not always with the same statistical significance.

#### *Systems biology analysis*

Proteins confidently identified were listed and analyzed in GeneDecks to detect significantly enriched pathways and GO biological functions (Table 3). Different

pathways and GO terms related with the innate immune response, antioxidant activity and lipid metabolism were found highly significant.

#### *Additional analysis*

Results from the additional analyses performed to validate the proteomics results are detailed in Table 1B.

OTC-supplemented pigs showed higher serum PON1 levels and total antioxidant activity as measured by FRAP (not statistically significant), and significantly lower levels of  $\alpha$ 1AG as detected by western blot. Additional evaluation of PON1 activity has been undertaken because in spot #484 also peptides of  $\alpha$ 1AG had been found; activity measurement showed numerically higher levels of PON1 in the supplemented group (Table 1B).

Serum Hp concentrations in the samples analyzed by 2DE DIGE were significantly lower in OTC-supplemented animals. No differences were found in the total IgG content.

## **4 Discussion**

In the present study we aimed at the identification of the systemic immune and metabolic effects of OTC supplementation through the analysis of the pig serum proteome in treated and untreated animals. We used serum as its composition is known to reflect the physiological status of the individual [26], and proteomic analysis because it allows for the analysis of multiple proteins. However, blood-derived samples are difficult to analyze due to their high dynamic range (10-12 orders of magnitude) and because of the presence of a low number of highly abundant proteins [27]. To allow detection of concentration differences in low abundant serum proteins three different

enrichment techniques were used. Due to the different underlying principles, the enrichment factor and resulting protein composition were highly dependent on the respective methods. This is well in line with previous reports, even though they partly used other prefractionation protocols [28]. All three methods in our experiments yielded enough protein to perform 2DE DIGE analysis. The Proteominer method yielded only spots of various immunoglobulin (Ig) chains. Similar differences concerning Ig chains are regularly found in many proteomics and transcriptomics studies, with unknown significance. Here, total immunoglobulin levels were not altered, as quantified by immunoassay.

Animals treated with OTC showed significant growth promotion and reduction in the acute phase protein SAA concentration compared to the control group. Proteomic analysis showed that growth promotion by OTC was accompanied with serum proteome changes largely consistent with down regulation of the inflammatory response. These responses are seen with the classic acute phase proteins, as well as with the HDL apolipoproteins CLU, ApoF and most likely also with PON1. Whereas the changes in the former two are unambiguous, in the spot of PON1 also peptides of  $\alpha$ 1AG were found, raising questions about the origin of the up regulation. Because quantification based on MS was not possible, biochemical serum analysis of PON1 activity was performed, and showed a numerical up regulation, whereas  $\alpha$ 1AG was significantly down regulated in serum by immunological analysis. Furthermore,  $\alpha$ 1AG was also down regulated in another spot (#515). Based on the above, we are fairly confident that PON1 (and not  $\alpha$ 1AG) is responsible for the up regulation found.

Concerning the acute phase proteins, OTC-supplemented pigs showed down regulated spots of Hp and  $\alpha$ 1AG. When analyzing all animals of the present experiment (n=27) by colorimetric assay, Hp level decrease in serum was marked, but not



statistically significant. However, including only the animals from the 2DE DIGE experiment (n=8), changes determined in this assay were significant, thus confirming our proteomic results. A similar trend was observed when comparing the intensity of the Hp alpha chain protein bands in Figure 1, which further supporting that OTC-treated animals' plasma contained lower levels of Hp. Significantly lower levels of serum  $\alpha$ 1AG were found in OTC-supplemented pigs by 2DE DIGE, and subsequently confirmed by immuno-blotting.  $\alpha$ 1AG is in most species a positive acute phase protein. Recent evidences suggest that  $\alpha$ 1AG is a negative acute phase protein in the pig during infection, whereas it has been observed that obese pigs from certain breeds show significantly higher serum levels of  $\alpha$ 1AG compared to lean counterparts [29]. In humans, the levels of serum  $\alpha$ 1AG rise with obesity, which is considered as a sign of overweight-associated low-grade inflammation [30]. Interestingly, levels of this protein have also been identified to be strongly inversely correlated with growth rate in swine in different studies, in the absence of confounding environmental, health and management factors [29,31]. It is therefore possible that different genetic backgrounds and inflammatory stimuli result in different responses of  $\alpha$ 1AG. In any case, the decrease in  $\alpha$ 1AG levels in the present study paralleled those of SAA and Hp, and lower levels of positive acute phase proteins are known to positively correlate with daily weight gain and feed efficiency [32]. In contrast to Hp and  $\alpha$ 1AG, SAA was measurable only by ELISA in serum and could not be identified by 2DE DIGE. This could be due to the unique multimeric nature of pig SAA [33], causing it to disperse over the gel into multiple small and hence undetectable spots. Based on the down regulation of the positive acute phase proteins Hp,  $\alpha$ 1AG, and SAA up regulation of the negative acute phase protein albumin was expected. No changes were observed in the main spot of albumin either in 1DE or 2DE gels, however, some smaller fragments of albumin were

found in lower or higher concentration in OTC-supplemented animals, for which we have no plausible explanation.

CLU, PON1 and ApoF are all HDL-related proteins which we found increased in OTC-supplemented piglets. This also fits with an anti-inflammatory response because HDL itself can be considered a negative acute phase reactant. However, this may not be the only explanation, because no other HDL proteins were found to be regulated. CLU (or apolipoprotein J) is a secreted chaperone with anti-apoptotic, antioxidant and anti-inflammatory functions that maintains fluid-epithelial interface homeostasis, thereby preventing the onset of inflammatory conditions [34]. Data relating CLU serum levels during obesity and systemic inflammation are conflicting: some studies claim up-regulation and a correlation with inflammatory markers [35] whereas in others either no clear relationship was found [36] or a down regulation was observed [37, 38]. A decrease in plasma CLU levels during weight reduction has been observed in humans [36] and dogs [39], which has been proposed to be an effect of the hypocaloric diet rather than being directly linked to weight loss [36]. Furthermore, CLU serum concentration is associated with those of other adipokines in relation to insulin sensitivity/hyperglycemia and lipid metabolism, but not to body fat mass and inflammation [40]. Altogether, these data suggest that CLU might be an indicator of the body response towards diet-induced oxidative stress. Indeed, there is recent molecular evidence for the protective role of CLU against high-fat diet-induced insulin resistance through the suppression of oxidative stress and inflammation [41]. The activities of CLU and PON1 are closely related within the HDL particle, being responsible for the protection of low density lipoproteins from oxidative modification [42]. PON1 is a negative acute phase reactant in pigs [24]. PON1 is an antioxidant protein whose main function appears to be the metabolism of toxic oxidized lipids of both low density

lipoprotein (LDL) particles as well as HDL particles [43]. Yet, this protein also exerts anti-inflammatory functions, such as directly suppressing macrophage pro-inflammatory responses [44]. Down regulation of PON1 levels is associated with exacerbation of obesity-related inflammation and oxidative stress [45]. We determined the enzymatic activity of PON1 in serum, and the total antioxidant activity, and both were numerically lower in control animals. Although not significant probably due to the low sample size, it suggested a link between inflammation and lipid oxidation, since the exhaustion of the enzymatic activity of PON1 has been related with the systemic low-grade inflammation produced by oxidative stress in coronary heart disease patients [46]. The third up-regulated apolipoprotein, ApoF, is also an atheroprotective protein, and involved in cholesterol transport between lipoproteins, and their levels are positively associated with a favorable proteomic signature for HDL [47].

It is concluded that the above findings on the acute phase proteins and HDL apolipoproteins can be correlated in a straight forward manner with OTC-supplementation causing a lower inflammatory state associated with decreased oxidative stress. This is much less straight forward with AHSG and HP-27. AHSG (also known as fetuin-A) is a pleiotropic molecule with metabolic and both anti- and pro-inflammatory roles [48]. Both AHSG under- and over regulation have been described to correlate with inflammation, the first being related with the acute phase reaction [49] and the latter with the development of obesity-related disorders [50]. Concerning AHSG, lower levels were found only with of two AHSG breakdown products, a 34 kDa C-terminal fragment, and a 15 kDa N-terminal fragment, but not with intact AHSG. The biological role of these smaller products is unclear.

Interestingly, in the OTC-supplemented pigs HP-27 was down regulated. Hibernation-associated plasma proteins (HP) were first identified in chipmunks as a

group of proteins produced in the liver with fluctuating serum levels, depending on the season. They appear at high concentration in the brain during hibernation [51]. However, they have been also identified in the plasma from non-hibernating mammals such as squirrels [52] and cattle [53, 54]. To our knowledge, this is the first report of the presence of HP proteins in pig serum. The exact function of HP proteins is unknown, but structurally homologous proteins have been shown to contribute to energy homeostasis, immunity, tumor cell apoptosis, and cell survival. It has been therefore proposed that an increase in brain HP proteins during hibernation may protect organisms from depressed metabolism and lethal diseases as observed in hibernators [51]. The molecular mechanisms of the metabolic rate depression occurring during hibernation include the suppression of energy-expensive cell functions, reprioritization of ATP use, and enhanced expression of protective, anti-oxidative mechanisms [55]. This is accompanied by a lipid-based metabolism, transient insulin resistance and hyperinsulinemia, but by none of the pathological features of metabolic syndrome [55]. In our case, the lower levels of HP-27 in OTC-supplemented animals might be interpreted as indicative of a metabolic switch by OTC to an energy-saving, antioxidant phenotype, similar to what happens in hibernators. Furthermore, pig HP-27 may depress appetite, as has been suggested for bovine HPs injected intracerebroventricularly in mice. Although the latter results have to be interpreted with caution [54], the lower HP-27 serum levels found in the OTC group here could be consistent with the observed increased appetite in OTC treated animals [5, 6]. Pro-inflammatory cytokines are thought to be responsible for (in)appetence, but the present results may be indicative of a new alternate pathway. However, it is entirely unclear whether or not inflammation and HP-27 abundance are linked. Further research should be performed to confirm the precise role of this relatively unknown protein.

452

453 In conclusion, in feed OTC supplementation of weaned piglets produced changes in the  
454 serum proteome largely consistent with an anti-inflammatory mechanism for OTC.  
455 These changes reflected a metabolic change to an energy-saving, growth-promoting  
456 phenotype associated with enhanced protection against lipid oxidation. The obtained  
457 results support the anti-inflammatory hypothesis as the main mechanism of action of  
458 AGP. The latter may also offer a plausible mechanism for the hitherto unexplained  
459 growth promotion by antibiotics observed in children [56, 13]. Finally, given that pigs  
460 are good models for humans for their great genetic and physiologic resemblance, the  
461 present results might also be relevant for human immunometabolic diseases such as  
462 obesity, type 2 diabetes and cardiovascular diseases [57].

463

#### 464 **Acknowledgements**

465 J.J. Cerón and K. De Backer are acknowledged for their help during results validation.  
466 Lotte Svare Holck is thanked for technical assistance with the Triton X-114 cloud point  
467 separation method. L. Soler acknowledges financial support by the Martín Alonso  
468 Escudero Foundation, Spain.

469

470 All authors declare no conflict of interest.

471

472

#### 473 **5 References**

474

475 [1] Hotamisligil, G.S., *Nature* 2006, *444*, 860-867.

476 [2] Mathis, D., Shoelson, S.E., *Nat. Rev. Immunol.* 2011, *11*, 81.

- 477 [3] Margioris, A.N., *Curr. Opin. Clin. Nutr. Metab. Care* 2009, *12*, 129-137.
- 478 [4] Niewold, T.A., in: Garnsworthy P.C., Wiseman J. (Eds.) *Recent Advances in Animal*  
 479 *Nutrition*, Nottingham University Press, Nottingham, 2010. pp. 191–205.
- 480 [5] Niewold, T.A., *Poult. Sci.* 2007, *86*, 605-609.
- 481 [6] Khadem, A., Soler, L., Everaert, N., Niewold, T.A., *Br. J. Nutr.* 2014, *112*, 1110-  
 482 1118.
- 483 [7] Dibner, J.J., Richards, J.D., *Poult. Sci.* 2005, *84*, 634–643.
- 484 [8] Hoyt, J.C., Robbins, R.A., *FEMS Microbiol. Lett.* 2001, *205*, 1-7.
- 485 [9] Dunston, C.R., Griffiths, H.R., Lambert, P.A., Staddon, S., Vernallis, A.B.,  
 486 *Proteomics* 2011, *11*, 42-51.
- 487 [10] Costa, E., Uwiera, R.R., Kastelic, J.P., Selinger, L.B., Inglis, G.D., *Gut Pathog.*  
 488 2011, *3*, 14.
- 489 [11] Bosi, P., Merialdi, G., Scandurra, S., Messori, S., Bardasi, L., Nisi, I., Russo, D.,  
 490 Casini, L., Trevisi, P., *J. Anim. Sci.* 2011, *89*, 4043-4053.
- 491 [12] Gonzalez, L.M., Moeser, A.J., Blikslager, A.T., *Transl. Res.* 2015, *166*, 12-27.
- 492 [13] Schwartz, B.S., Pollak, J., Bailey-Davis, L., Hirsch, A.G., Cosgrove, S.E., Nau, C.,  
 493 Kress, A.M., Glass, T.A., Bandeen-Roche, K., *Int. J. Obes. (Lond.)* 2015, doi:  
 494 10.1038/ijo.2015.218. [Epub ahead of print]
- 495 [14] Soler, L., Gutiérrez, A., Martínez-Subiela, S., Ceron, J.J., *Vet. J.* 2011, *190*, e7-11.
- 496 [15] Boschetti, E., Righetti, P.G., *J. Prot.* 2008, *71*, 255-264.
- 497 [16] Miller, I., *Methods Mol. Biol.* 2012, *854*, 373-396.
- 498 [17] Jessen, F., Wulff, T., *Anal. Biochem.* 2015, *485*, 11-17.
- 499 [18] Bradford, M.M., *Anal. Biochem.* 1976, *72*, 248-254.
- 500 [19] Miller, I., Friedlein, A., Tsangaris, G., Maris, A., Fountoulakis, M., Gemeiner, M.,  
 501 *Proteomics* 2004, *4*, 3227-3234.

502 [20] Miller, I., Gemeiner, M., *Electrophoresis* 1992, *13*, 450-453.

503 [21] Marco-Ramell, A., Hummel, K., Razzazi-Fazeli, E., Bassols, A., Miller, I.,

504 *Electrophoresis* 2015, *36*, 543-551.

505 [22] Stelzer, G., Inger, A., Olender, T., Iny-Stein, T., Dalah, I., Harel, A., Safran, M.,

506 Lancet, D., *OMICS* 2009, *13*, 477-487.

507 [23] Ceciliani, F., Pocacqua, V., Miranda-Ribera, A., Bronzo, V., Lecchi, C., Sartorelli,

508 P., *Vet. Immunol. Immunopathol.* 2007, *116*, 145-152.

509 [24] Escribano, D., Tvarijonaviciute, A., Tecles, F., Cerón J.J., *Vet. Immunol.*

510 *Immunopathol.* 2015, *163*, 210-215.

511 [25] Benzie, I.F., Strain, J.J., *Anal. Biochem.* 1996, *239*, 70-76.

512 [26] Miller, I., Wait, R., Sipos, W., Gemeiner, M., *Res. Vet. Sci.* 2009, *86*, 362-367.

513 [27] Anderson, N.L., Anderson, N.G., *Mol. Cell. Proteomics* 2002, *1*, 845-867.

514 [28] Fernández, C., Santos, H.M., Ruíz-Romero, C., Blanco, F.J., Capelo-Martínez, J.L.,

515 *Electrophoresis* 2011, *32*, 2966-2974.

516 [29] Rødgaard T, Skovgaard K, Stagsted J, Heegaard PM. *Cell Reprogram.* 2013

517 *Jun;15(3):185-94.*

518 [30] Maachi M1, Piéroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J,

519 Bastard JP. *Int J Obes Relat Metab Disord.* 2004 Aug;28(8):993-7.

520 [31] Heegaard, P.M., Miller, I., Sorensen, N.S., Soerensen, K.E., Skovgaard, K., *PLoS*

521 *One* 2013, *8*, e68110.

522 [32] Clapperton, M., Diack, A.B., Matika, O., Glass, E.J., Gladney, C.D., Mellencamp,

523 M.A., Hoste, A., Bishop, S.C., *Genet. Sel. Evol.* 2009, *30*, 41:54.

524 [33] Soler, L., Molenaar, A., Merola, N., Eckersall, P., Gutiérrez, A., Cerón, J., Mulero,

525 V., Niewold, T., *J. Theor. Biol.* 2013, *317*, 119-125.

526 [34] Jones, S.E., Jomary, C., *Int. J. Biochem. Cell Biol.* 2002, *34*: 427-431.

527 [35] Won, J.C., Park, C.Y., Oh, S.W., Lee, E.S., Youn, B.S., Kim, M.S., *PLoS One*  
528 2014, 9, e103351.

529 [36] Arnold, T., Brandlhofer, S., Vrtikapa, K., Stangl, H., Hermann, M., Zwiauer, K.,  
530 Mangge, H., Karwautz, A., Huemer, J., Koller, D., Schneider, W.J., Strobl, W.,  
531 *Pediatr. Res.* 2011, 69, 237-242.

532 [37] Hoofnagle, A.N., Wu, M., Gosmanova, A.K., Becker, J.O., Wijsman, E.M.,  
533 Brunzell, J.D., Kahn, S.E., Knopp, R.H., Lyons, T.J., Heinecke, J.W., *Arterioscler.*  
534 *Thromb. Vasc. Biol.* 2010, 30, 2528-2534.

535 [38] Martos-Moreno, G.Á., Sackmann-Sala, L., Barrios, V., Berrymann, D.E., Okada,  
536 S., Argente, J., and Kopchick, J.J., *Int J Pediatr. Endocrinol.* 2014, 2014, 9.

537 [39] Tvarijonaviciute, A., Gutiérrez, A.M., Miller, I., Razzazi-Fazeli, E., Tecles, F.,  
538 Ceron, J.J., *Domest. Anim. Endocrinol.* 2012, 43, 271-277.

539 [40] Flehmig, G., Scholz, M., Klötting, N., Fasshauer, M., Tönjes, A., Stumvoll, M.,  
540 Youn, B.S., Blüher, M., *PLoS One* 2014, 9, e99785.

541 [40] Kwon, M.J., Ju, T.J., Heo, J.Y., Kim, Y.W., Kim, J.Y., Won, K.C., Kim, J.R., Bae,  
542 Y.K., Park, I.S., Min, B.H., Lee, I.K., Park, S.Y., *Endocrinol.* 2014, 155, 2089-  
543 2101.

544 [42] Kujiraoka, T., Hattori, H., Miwa, Y., Ishihara, M., Ueno, T., Ishii, J., Tsuji, M.,  
545 Iwasaki, T., Sasaguri, Y., Fujioka, T., Saito, S., Tsushima, M., Maruyama, T.,  
546 Miller, I.P., Miller, N.E., Egashira, T., 2006. *J. Atheroscler. Thromb.* 2006, 13, 314-  
547 322.

548 [43] Costa, L.G., Vitalone, A., Cole, T.B., Furlong, C.E., *Biochem. Pharmacol.* 2005,  
549 69, 541-550.

550 [44] Aharoni, S., Aviram, M., Fuhrman, B., *Atheroscler.* 2013, 228, 353-361.



551 [45] Krzystek-Korpacka, M., Patryn, E., Hotowy, K., Czapińska, E., Majda, J.,  
552 Kustrzeba-Wójcicka, I., Noczyńska, A., Gamian, A., *Adv. Clin. Exp. Med.* 2013, 22,  
553 229-236.

554 [46] Kotur-Stevuljevic, J., Spasic, S., Jelic-Ivanovic, Z., Spasojevic-Kalimanovska, V.,  
555 Stefanovic, A., Vujovic, A., Memon, L., Kalimanovska-Ostric, D., *Clin. Biochem.*  
556 2008, 41, 1067-1073.

557 [47] Lagor, W.R., Fields, D.W., Khetarpal, S.A., Kumaravel, A., Lin, W., Weintraub,  
558 N., Wu, K., Hamm-Alvarez, S.F., Drazul-Schrader, D., de la Llera-Moya, M.,  
559 Rothblat, G.H., Rader, D.J., *PLoS One* 2012, 7, e31616.

560 [48] Arnaud, P., and Kalabay, L., *Diabetes Metab. Res. Rev.* 2002, 18, 311-314.

561 [49] Lebreton, J.P., Joisel, F., Raoult, J.P., Lannuzel, B., Rogez, J.P., Humbert, G., *J.*  
562 *Clin. Invest.* 1979, 64, 1118-1129.

563 [50] Trepanowski, J.F., Mey, J., Varady, K.A., *Int. J. Obes. (Lond.)* 2015, 39, 734-741.

564 [51] Kondo, N., *Cold Spring Harb. Symp. Quant. Biol.* 2007, 72, 607-613.

565 [52] Ono, M., Kojima-Kawagoe, M., Kondo, N., Shiba, T., Takamatsu, N., *Gene* 2003,  
566 302, 193-199.

567 [53] Wait, R., Miller, I., Eberini, I., Cairolì, F., Veronesi, C., Battocchio, M., Gemeiner,  
568 M., Gianazza, E., *Electrophoresis* 2002, 23: 3418-3427.

569 [54] Seldin, M.M., Byerly, M.S., Petersen, P.S., Swanson, R., Balkema-Buschmann, A.,  
570 Groschup, M.H., Wong, G.W., *J. Exp. Biol.* 2014, 217, 2667-2679.

571 [55] Martin, S.L., *Diab. Vasc. Dis. Res.* 2008, 5, 76-81.

572 [56] Gough, E.K., Moodie, E.E., Prendergast, A.J., Johnson, S.M., Humphrey, J.H.,  
573 Stoltzfus, R.J., Walker, A.S., Trehan, I., Gibb, D.M., Goto, R., Tahan, S., de Morais,  
574 M.B., Manges, A.R., *Brit. Med. J.* 2014, 348, g2267.

575 [57] Esser, N., Paquot, N., Scheen, A.J., *Expert Opin. Investig. Drugs* 2014, 25, 1-25.

576

**Table 1. Animal data**

A. Weight gain and acute phase protein of pigs.

B. Validation of selected factors identified by proteomic analysis by other methods.

**Table 2. Proteins differentially regulated in OTC-treated pigs' serum and identified by MALDI-TOF/TOF mass spectrometry.** Indicated by asterisk (\*) is spot #715, included (near significant) to complete the information about clusterin spot #758, as both spots belong to different clusterin chains ( $\alpha$ , and  $\beta$  respectively).

**Table 3.** GeneDecks pathway analysis of proteins differentially regulated in OTC-treated animals.

**Legends to figures**

**Figure 1. SDS–PAGE protein profiles of serum samples before (A, Coomassie staining) and after (B-D, silver-staining) depletion treatments.**

(A) Protein pattern of some whole serum samples from control and OTC-treated animals is shown. In B and C the protein profile of fractions (bound and eluted) obtained after Proteominer and ProteaPrep treatment is shown, respectively. Asterisks indicate samples later analyzed by 2DE DIGE. In D the protein profile before (1) and after (2,3) Triton X-114 cloud point separation of two control pig serum samples is shown.

**Figure 2. 2DE of serum samples and depleted fractions**

602 Representative 2DE gel images of whole serum proteins (A) and after  
603 enrichment/depletion treatment with Proteominer (B), ProteaPrep (C) and Triton X-114  
604 cloud point separation (D). Protein spots significantly regulated after OTC treatment  
605 (see Table 2) are indicated. Italic numbers (A; spots #328-331) represented unregulated  
606 intact AHSG chains. Brackets indicate the position of the series of related CLU chains  
607 (D).

608

609 **Supplemental table 1:** Peptide table of mass spectrometric protein identification (Carb:  
610 Carbamidomethylation on cysteine, Ox: Oxidation on methionine)